

Reactions of Asparagine in Phosphate Buffer

By T. J. Fitzpatrick, E. A. Talley and W. L. Porter

Eastern Utilization Research and Development Division, Agricultural Research Service,
United States Department of Agriculture, Philadelphia 18, Pennsylvania, U.S.A.

While investigating procedures for the analysis of glutamine and asparagine in natural products,¹ L-asparagine was heated in phosphate buffer at 100°C. as in the glutamine method described by Pucher and Vickery,² and Hamilton.³ These investigators had reported no interference by asparagine in the glutamine determination. However, it was found in the present study by means of ion exchange techniques, that a definite set of reactions did occur under their conditions. These reactions, though minor in yield, were sufficient to produce high values for glutamine when the asparagine-glutamine ratio was one or more, as in potato extracts. Non-ninhydrin active fumaramic acid, previously reported,¹ and two ninhydrin positive compounds were formed in low but measurable concentrations which were isolated and identified.

Analysis, by means of an automatic amino-acid analyser,⁴ of a solution resulting from heating L-asparagine for 90 minutes at 100°C. in phosphate buffer³ showed the production of two unknown peaks as well as the residual asparagine. The first of these peaks present in the larger amount was tentatively identified as aspartic acid by means of the effluent volume. This portion of the column effluent was isolated and desalted by the method of Drèze, *et al.*⁵ Paper chromatography on Whatman No. 1 paper using n-butanol-glacial acetic acid-water (8:2:2) gave an R_F value of 0.24 and using pyridine-water (65:35) gave an R_F value of 0.40 for both the unknown compound and known aspartic acid. These values (in both solvents) agreed with Berry, *et al.*⁶ and indicate the identity of the isolated material to be aspartic acid.

The second unknown reaction product eluted in a more basic position than L-asparagine and produced a peak on the analyser chart in the position of isoleucine. It was found, by experimental means, that as much or more of this unknown was produced by heating 7 hours at 120°C. in an autoclave as was produced by heating 24–35 hours at 100°C. at atmospheric pressure. Therefore, to prepare a sufficient quantity of the unknown for identification, 7 g. of L-asparagine was heated in pH 6.7 phosphate buffer in a loosely covered flask for 7 hours at 120°C. in the autoclave at 15 p.s.i. and the desired fraction was isolated by means of a large preparative ion exchange column.⁷ This unknown fraction was desalted by the method of Buchanan⁸ and the pyridine removed by means of a rotary vacuum evaporator. Initial crystallisation was from 70% isopropanol. Subsequent recrystallisations from water yielded 150 mg. (Found: C, 36.71; H, 7.04; N, 21.10; mol. wt. 135.3 (freezing point depression in water); Amino N (Van Slyke), 10.70; Amide N (HCl Hydrolysis), 10.80; Biuret test, positive. Calculated for

$C_4H_8N_2O_3$: C, 36.36; H, 6.10; N, 21.21; mol. wt., 132.12; Amino N, 10.60; Amide N, 10.60%). These data indicated the material to be either an isomer or a peptide of L-asparagine. Negative hydrolysis with carboxypeptidase eliminated the possibility of an α -peptide. A sample of L-isoasparagine⁹ had the same mobility as the unknown on the amino-acid analyser, the infrared spectra were identical, but X-ray diffraction indicated a definite difference in structure.

DL-Isoasparagine was prepared by dissolving dry N-carbobenzoxy-DL-aspartic acid in acetic anhydride, treating the resulting anhydride with ammonium hydroxide and removing the carbobenzoxy group by hydrogenolysis according to the method of Bergmann and Zervas.¹⁰ Infrared absorption values and X-ray diffraction patterns for the synthetic DL-isoasparagine and the isolated material were identical. No optical rotation could be measured. Therefore, it was concluded that the isolated compound was DL-isoasparagine (DL-3-aminosuccinamic acid).

The results indicate that L-asparagine heated in pH 6.7 phosphate buffer at 100°C. forms fumaramic and aspartic acids. Fumaramic acid gradually adds free ammonia to the double bond to form DL-isoasparagine and DL-asparagine. Both asparagines are gradually deamidated on prolonged heating to form as a final product aspartic acid. DL-isoasparagine reaches a maximum of 3.5%, based upon the asparagine starting material in 35 hours at 100°C. Approximately this same maximum is reached at 7 hours at 120°C. After 192 hours at 100°C. aspartic acid constitutes 65% of the reaction mixture. Analysis of a glutamine-asparagine mixture (heated in phosphate buffer for 90 minutes at 100°C.) would decrease the asparagine value by about 3.5% to 4% and, therefore, produce an error in the glutamine determination.

References

- 1 Talley, E. A., Fitzpatrick, T. J. & Porter, W. L., *J. Amer. chem. Soc.*, 1959, **81**, 174
- 2 Pucher, G. W. & Vickery, H. B., *Ind. Engng Chem., Anal. Ed.*, 1940, **12**, 27
- 3 Hamilton, P. B., *J. biol. Chem.*, 1945, **158**, 375
- 4 Spackman, D. H., Stein, W. H. & Moore, S., *Anal. Chem.*, 1958, **30**, 1190
- 5 Drèze, A., Moore, S. & Bigwood, E. J., *Anal. Chim. Acta*, 1954, **11**, 554
- 6 Berry, H. K., Sutton, H. E., Cain, L. & Berry, J. S., *Univ. of Texas Bull.*, 1951, No. 5109, 22
- 7 Talley, E. A. & Porter, W. L., *J. Chromatography*, 1960, **3**, 434
- 8 Buchanan, C. L., *Anal. Chem.*, 1957, **29**, 1877
- 9 Gratefully received from E. Sondheimer, N.Y. State College of Forestry
- 10 Bergmann, M. & Zervas, L., *Ber. dtsh. chem. Ges.*, 1932, **65**, 1196